ISOLATION OF THE GLYCOPROTEIN OF VESICULAR STOMATITIS VIRUS AND ITS BINDING TO CELL SURFACES

by

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SECTION II

LITERATURE REVIEW

LITERATURE REVIEW

General Features of Viral Attachment

The initial step in viral infection is the attachment of the virus to the host cell. The nature of this attachment and the necessary requirements for the attachment have been the subject of many studies. Host range determinations of various viruses were probably the first studies characterizing the cellular specificity of viral attachment. Through these studies, it was found that most viruses possessed a narrow host range and could only infect cells from certain species of animals. For example, murine leukemia virus, a retrovirus, can only infect murine tissues or cell lines (38) and, likewise, poliovirus will usually only infect primate tissue (48). There are other viruses, like vesicular stomatitis virus (VSV), that have a relatively broad host range. VSV will commonly infect bovine and equine species and most established cell lines except non-stimulated T-cells and domestic rabbit kidney and cornea cells (25, 104, 133). Because most viruses have discrete host ranges, specific interactions must occur between a susceptible cell and the infecting virus that do not occur with a resistant cell. At first, the specific interaction between virus and host cell was believed to only involve the process of viral attachment but further studies showed viral penetration, uncoating, replication, assembly, and release might also be limiting stages for viral infection (127). Since viral attachment is the initial interaction of a virus with a cell, it is of particular interest in the study of the infectious process.

The manner by which viruses attach to the surface of susceptible host cells is not specifically known. It has been suggested that the interaction of specific host-virus receptors could explain the specificity and physical features of viral attachment. Essentially, a viral component functions as

the viral receptor which directly interacts with a cell surface moeity (cell receptor). This interaction must be strong enough to maintain the virion at the cell surface to allow penetration of the virus into the cell. Specific proteins or glycoproteins of viruses have been shown to function as viral components of attachment (80). Unlike bacteriophages, most animal viruses do not have structural components that can easily be identified with the process of viral attachment. The presence of virion fibers, which share functional receptor properties with bacteriophages, appears to be restricted to the members of the adenovirus group. In addition to the difficulty in identifying viral components associated with attachment, identification of the cellular receptor also has been difficult due to the large number of proteins and glycoproteins on the surface of the eukaryotic cell. In some cases, erythrocytes, which have relatively simple cell surfaces, have been used to identify the cellular receptors for myxoviruses and adenoviruses (80, 110).

The kinetics of virus attachment have been analyzed for numerous animal viruses. In kinetic experiments, quantitation of virus binding has been commonly determined by three methods: infectious assays, hemagglutination assays, or measurements of the attachment of radiolabeled virions (135). Most often, the amount of virus which bound to host cells was determined by calculating the difference between the amount of virus initially introduced to a cell population and the virus which failed to bind. The quantities of virus initially added and that which failed to attach were determined by quantitating infectious particles or hemagglutination units by the plaque assay or hemagglutination assay, respectively. The use of radiolabeled virions, developed more recently, provides a more direct and accurate measurement of virion attachment. Unlike the plaque assay method, which is limited to infectious particles and the hemagglutination assay that

is quite insensitive, the methods using radiolabeled virions measure attachment of total physical particles and the technique is very sensitive.

The number of viruses which bind per cell or the number of cellular receptors per cell has been determined for various virus-cell systems. The maximum number of infectious coxsackievirus B3 that bound to HeLa cells was found to be 200-300 plaque forming units per cell. This quantity of infectious virus was equivalent to approximately 1×10^5 physical particles per cell (27). Similarly, saturating levels of rhinovirus attached to HeLa cells have been $1\text{-}2 \times 10^4$ virion particles per cell (80) and approximately 1×10^4 receptor sites per HeLa cell were estimated for adenovirus type 2 (112). Approximately $0.3\text{-}1 \times 10^4$ poliovirus particles attach per HeLa cell while 10-100 times that amount binds to human fibroblasts (80, 88). In the case of poliovirus, the area covered by 10^4 picornavirus particles is only 1% of the surface of the cell membrane (79). In all cases where saturation has been reached, the saturation was independent of cell surface space and not a result of spatial limitations. Contrary to the viruses previously mentioned, the binding of some viruses, paramyxoviruses and myxoviruses (80), is not saturable on their host cell which may be a reflection of the nature of the cellular receptor. The cellular receptor for myxoviruses or paramyxoviruses could possibly be a cellular component which is very abundant on the cell surface or the cellular receptor for the virus may be a specific moiety, i.e. carbohydrate, which is common to a large number of cell surface proteins. Another possible explanation for being unable to saturate viral attachment is myxoviruses and paramyxoviruses may have a number of binding sites on the cell that are both specific and nonspecific.

The rate of virion attachment depends on the concentration of cell surface receptors (i.e. the number of cells per milliliter). The efficiency of attachment of virions which collide with a cell is very low and the binding

of virus will follow first order kinetics if less than saturating levels of receptors were used (80). Various physiological factors such as pH, temperature, presence of divalent cations, and ionic strength of the binding environment also effect the rate of attachment of virions to host cell.

The pH optima for binding of virions has been determined for many virus-cell systems. Adenovirus and myxoviruses bind erythrocytes optimally in a broad pH range from pH 5.5 to 8.7 (55,102). However, echovirus type 7 optimally attaches to erythrocyte membranes in a narrow range between pH 5 and 6 (108), and coxsackievirus B4 optimally attaches to HeLa cells between pH 3 and 3.5 (26).

Myxoviruses (1), polyoma virus (24), equine rhinovirus (79), and cardioviruses (mengo, mengo encephalomyocarditis, Col SK, or encephalomyocarditis viruses) have been shown to attach rapidly to host cells and the attachment is not temperature sensitive (51, 64, 76). However, the attachment of most other viruses is less efficient at low temperatures. Coxsackievirus B, arboviruses, and adenovirus type 2 show a termperature dependency in their rate of attachment (80, 107). The binding of poliovirus type 1 to monolayers of HeLa cells showed a 10-fold reduction in rate of binding at $1^{o}C$ relative to $37^{o}C$ (49). Lonberg-Holm and Korant (79), also found poliovirus type 1 and 2, as well as rhinovirus type 2 and 14, to have a rate of attachment to suspended HeLa cells which increased by a factor of 2-3 times per $10^{o}C$ increase in a temperature range of 10^{o}-$37^{o}C$. The decreased rates of attachment at lower temperatures was apparently not based on either the decreased collision frequency between virus and suspended cell or an increase in viscosity of the suspension media (80). Allison and Valentine (1) developed an equation which corrects for the effect of temperature on the maximum collision frequency of the virus and cell. However, the observed temperature dependent decrease in rate of attachment mentioned was much

greater than what was predicted by the Allison and Valentine equation (80). The temperature sensitivity of attachment may be a reflection of a cellular metabolic step or a conformational change in either virions or the cell membrane which is required for a firm virus-cell complex to form.

The presence of divalent cations during viral attachment may infleunce the rate of attachment. Unfortunately, the manner in which most of the experiments were conducted do not provide definite conclusions as to the role that divalent cations may have for attachment. For example, Bachtold et al. (3) reported that poliovirus attachment was enhanced by magnesium and calcium but the monkey cells used had been trypsinized and trypsin has been shown to alter the number and characteristics of cellular receptors (80). Other studies with nontrypsinized cells indicated divalent cations were not required for attachment of poliovirus (49, 134). Many investigators have shown that treatment of cells with sodium ethylene-diamine tetraacetate (EDTA) inhibited binding of coxsackievirus and human rhinovirus (79, 84). EDTA will chelate divalent cations from the surface of the cell but it is possible that such treatment may have denatured or altered the proteins of the cell membrane. If, in fact, divalent cations enhance the attachment of certain viruses, it would provide a reasonable mechanism by which initial binding occurs. The positively charged ions could mediate between the negatively charged cell surface and negatively charged virion. It would provide a charge balance which could, therefore, allow the virus to interact with the cell electrostatically and to eventually promote a stronger attachment that allows penetration of the virion.

The Viral Components Involved in Attachment

For many viruses, the various proteins which compose the virion have been characterized and in some cases, specific functions associated with

viral replication, have been attributed to particular viral proteins. In particular, such studies have determined that one or two structural proteins of the virus function as the viral component which directly interacts with the host cell surface during attachment.

Influenza virus, a well characterized RNA enveloped virus, has been extensively used as a model for studying the stages of viral attachment (80). Two antigenically distinct proteins of influenza virus apparently influence virion attachment. Both are envelope glycoproteins which protrude from the virion surface but each have a different and specific function in binding (143, 147). One protein, the hemagglutinin, is responsible for viral hemagglutination of erythrocytes and viral attachment to susceptible host cells (55). Antibody directed against the hemagglutinin prevents hemagglutination, virion attachment, and neutralizes viral infection (80). The second glycoprotein associated with the host-virus interaction is the viral neuraminidase. This enzyme is responsible for the receptor destroying activity of the virus which results in the hydrolysis of the host cell surface receptor glycoprotein and leads to the elution of the virus from host cells or erythrocytes (55). However, it is not known what, if any, role the neuraminidase plays in the infectious disease process.

Adenoviruses are non-enveloped DNA viruses which have icosahedral symmetry (111). At the 12 vertices of the icosahedron an elongated antenna-like structure called the fiber can be seen projecting from the cell (106). The fiber has been identified as the attachment structure for adenovirus particles (121). Purified fiber, added in excess to HeLa cells, can prevent the attachment of intact adenovirus. Likewise, an excess of virions prevented fiber molecules from binding to cells (62, 112). Antibody directed against the fiber inhibited virion attachment as well as the fiber attachment (55).

Between 10^5-10^6 fiber moecules were found to bind per HeLa cell. This quantity is 10-100 times greater than the number of intact virions would bind per HeLa cell (112). Since the fiber competes with the virion receptor, the numerical difference suggests the fiber reaches cellular receptors not available to the large virion or more than one fiber can bind per virion.

The viral receptors identified for enveloped viruses largely are glycoproteins which are constituents of the viral envelope. For example, murine leukemia virions possess an envelope glycoprotein (gp71) which has the ability to bind to host cell surfaces (30). Likewise, the only glycoprotein of VSV is associated with viral attachment (18, 19). Glycoproteins of arboviruses and paramyxoviruses also have been designated as the viral component for attachment (12, 129). For non-enveloped viruses, the identification of the viral receptor material has not been as successful. One protein of poliovirus, VP4 (6,000 daltons), is suspected to function as the viral receptor (14, 80, 109) although it is not clear whether the VP4 protein is the only viral component responsible for attachment. Bolen and Consigli have investigated the specific and nonspecific adsorption of polyoma virus to mouse kidney cells (11). Their most recent studies have suggested that in polyoma virus, a subspecies of VP1 may be responsible for viral attachment and hemagglutination. They also have preliminary evidence that the viral component for hemagglutination and viral infection may be two separate subspecies of VP1 (Bolen, personal communication).

Cellular Receptors Involved in Attachment of Viruses

Due to the nature and complexity of the host cell surface, the identification of host cell receptors has only been studied in a few virus-host cell systems. Although a number of studies have investigated the viral aspects of attachment, little is presently known of the cellular involvement

in the attachment process. This is possibly due to the present lack of suitable techniques to identify, isolate, and chemically characterize surface membrane protein(s) which interact with a virus. Despite the complexity of identifying the cellular receptor, the cellular receptor for those viruses which hemagglutinate erythrocytes, e.g. myxoviruses, adenoviruses, and arboviruses, have been characterized fairly well due to the simplistic nature of the surfaces of red blood cells (55). For those non-hemagglutinating viruses, however, only indirect indications of the composition of the cell proteins associated with viral binding is available (80).

The identification of the viral receptor of erythrocytes that bind myxoviruses was one of the early monumental discoveries in virology. It first began when Hirst et al. (46) discovered that influenza viruses could hemagglutinate red blood cells. Fowl erythrocytes treated with neuraminidase were shown to be unable to bind the virion and this observation suggested that sialic acid of a glycoprotein on the cell surface medidated viral attachment (57). It was soon found that glycoproteins containing sialic acid, which were purified from serum or respiratory fluid, bound strongly to the virion and prevented the virion from binding to the host cell (55). A protein, to which influenza was shown to selectively attach, was extracted from erythrocytes with 50% hot phenol (68) or with lithium diiodosalicylate and 50% hot phenol (81). Chemical analysis of the extracted material showed the receptor to be a sialoglycoprotein with an apparent molecular weight of 55,000 daltons (54, 55, 68). This sialoglycoprotein contained the majority of the N-acetylneuramic acid of the erythrocyte membrane and the sialic acid was attached at the terminal end of the oligosaccharide (23, 29, 145). Interestingly, this cellular receptor also contained the determinants for the M and N blood groups (54, 68).

The discovery and characterization of the influenza cellular receptor explained earlier studies on the kinetics of influenza binding, i.e. the fairly broad host range of myxoviruses could be a reflection of the abundance of sialic acid in most cells (125). The temperature independence of binding by myxoviruses has also been attributed to the virus binding to the sialic acid proteins of the cell (80). The sufficient quantity of neuramic acid on the cell surface proteins can also explain why viral binding was never found to be totally saturable.

A detailed mechanism of attachment for myxoviruses has been proposed. Some ionizable amino group of the viral hemagglutinin, as indicated by the pH dependency for attachment (55, 102), electrostatically interacts with the terminal sialic acid of the glycoprotein identified as the cell receptor (80). This initial interaction would facilitate a stronger interaction between the hemagglutinin and the peptide portion of the cellular glycoprotein (145) which would then lead to the uptake (28) of the virion into the cytoplasm. Since the neuraminidase does not enhance the penetration of the virion into the cell (55), it may act as a protective mechanism for the virus. If the virus attached to a resistant cell and was not immediately engulfed into the cell, this would allow time for the viral neuraminidase to cleave the cellular receptor. This action would prevent the virus from entering an unfavorable environment and might allow the virus to attach to a susceptible host cell. Evidence also indicates that this reversible mechanism of viral attachment may be employed by paramyxoviruses attaching to their host cells (56, 128).

The identification of a cellular receptor for adenovirus has been accomplished. Initial studies indicate that antibody directed against the virion fiber protein prevented viral attachment and hemagglutination (80). Treating HeLa and KB cells with the proteolytic enzyme, subtilin, prevented

viral attachment, but the amount of virus binding was enhanced when cells were treated with trypsin, chymotrypsin, or neuraminidase. These latter enzymatic treatments probably increased the density of the cellular receptor or exposed more receptors thus increasing the amount of virus binding (112), although why subtilin destroys the cellular receptor is not known. The cellular receptor for adenovirus has been solubilized and partially purified by treating monkey erythrocytes with deoxycholate (101). The cellular receptor (40,000 daltons) was shown not to interact with myxoviruses and was resistant to neuraminidase that suggested that the adenovirus receptor differed from the cell surface glycoproteins which bind myxoviruses (82).

Based on the effects of pH and various chemicals on the viral attachment, a model has also been proposed for the attachment of adenovirus to host cells. The virion initially interacts with the host cell through one or two fiber structures binding to the cellular receptor (112). It is believed that an arginine molecule on the fiber protein forms a salt bond with the side chain of an aspartate or glutamate molecule on the 40,000 dalton cellular receptor (102). The virion attachment is strengthened by the apposition of additional cell receptors which will bind to the other available fibers (110). The virion is then engulfed by the plasma membrane and phagocytized into the cytoplasm of the host cell (28).

More extensive investigations and more sophisticated techniques in membrane biochemistry need to be developed in order to identify other viral receptors in the complicated cell plasma membrane. In recent years, the molecular biology of cell membranes has advanced to the point that many new techniques can be applied to determining the nature of cellular receptors for viruses. Recent advances in the study of murine leukemia virus (MuLV) present an example of this. The MuLV system is interesting because of the extremely limited host range of the virus. The virus will only infect

murine derived cells and MuLV is capable of transforming murine thymic cells (38). The cell surface binding sites, specific for the MuLV virions, are found in high concentrations on the thymic lymphoma cells which are induced by this virus, but are detectable at only low concentrations (if at all) in several non-thymic leukemias or normal thymocyte cells (4, 83). To better understand this phenomena, researchers have used purified preparations of the viral receptor (the envelope glycoprotein, gp71) to study the initial adsorption step in a productive viral infection (30, 39, 65, 144). Adding radiolabeled gp71 to various cell lines, Weissman et al., (144) also showed that gp71 bound in high quantities to thymic lymphomas and in low quantities to non-lymphomic cells. The binding of gp71 was rapid and approximately 5×10^5 molecules of gp71 saturated each cell within 25 minutes (30). Prior addition of intact virus or unlabeled gp71 would inhibit the binding of labeled gp71 to the cell surface (30, 39, 65). Attachment was temperature dependent such that an increase in temperature would increase the quantity of gp71 bound (30, 39, 65) and the maximum amount of binding occurred at a pH of 6 to 7 (30). Also, the amount of binding of gp71 increased as the concentration of calcium ions increased although magnesium or manganese did not enhance the binding (30, 65). When the isolated gp71 was first reacted with antiserum directed against the gp71, no binding could be detected (30).

The characteristics of the binding of the isolated gp71 have suggested the viral glycoprotein shares the cellular receptor with the intact virion. This has allowed investigators to use gp71 as a probe for the cellular receptor for MuLV. Cell membrane preparations were shown to have lost their receptor activity after being treated with the proteolytic enzymes trypsin, papain, and chymotrypsin. Neuraminidase or phospholipase A treatment did not affect the ability of the membrane to bind gp71, but binding was very sensitive to phospholipase C. The data suggested the cellular receptor

component or complex contains a lipoprotein (65). Isolation of this lipprotein has yet to be accomplished.

Investigations with other viruses and their cell surface receptors have produced only indirect evidence as to the composition of the cell surface receptor. For example, the cell receptor for poliovirus is believed to contain a lipoprotein since poliovirus attachment is affected by treating cellular membranes with ether or chloroform. However, poliovirus receptors are also moderately sensitive to trypsin and chymotrypsin and preincubation of cell membrane preparations with lipase did not reduce poliovirus binding (49, 148). Treating host cells with formaldehyde, periodate, or disulfide reducing agents did not affect viral attachment (47, 80). Since the use of hydrolytic enzymes is, at best, limited as a means to identify and characterize host cell surface receptors associated with virion adsorption, a considerable amount of additional information remains to be obtained. An extensive review of these rather indirect approaches to identify host receptors for virion attachment has been written by Lonberg-Holm and Philipson (80).

General Properties of Vesicular Stomatitis Virus

Numerous structural and biochemical studies have been conducted on vesicular stomatitis virus (VSV). Like other rhabdoviruses, VSV has a bullet-shaped morphology with one end appearing flat and the other end appearing as a tapered sphere. This cylindrical infectious virion is typically 180 ± 10nm in length and 65 ± 10nm in diameter (22, 52, 75). Another distinctive characteristic of these viruses is the presence of a lipoprotein envelope which has peplomers, or spikes, uniformly spaced throughout the entire surface of the virion. The protruding spikes are 10nm long and appear to consist of hollow knobs at the end of short stalks (20, 52). Wound inside the virion envelope is a helical ribonucleic

acid-protein complex. Negatively-stained preparations of the virus observed by electron microscopy often give the nucleoprotein complex a striated appearance (100). As is found in other rhabdoviruses, the nucleic acid of VSV is a single molecule of single-stranded RNA (5, 10). The overall chemical composition of the Indiana serotype of VSV virions, which was used in the following studies, includes 3% ribonucleic, acid, 64% protein, 13% carbohydrate, and 20% lipid (86).

The RNA genome, based on sedimentation analysis and direct measurements of electron micrographs of isolated RNA, has a molecular weight of 3.8×10^6 daltons (15, 71, 115) and this size genome has the capacity to code for all five viral proteins which compose the major proteins of the VSV virion (113). As is found in myxoviruses and paramyxoviruses, the RNA strand is designated as the negative strand because the RNA genome is antipolar to the viral mRNA and, therefore, is unable to directly serve as a template for viral-directed protein synthesis (5, 140). The VSV virion is composed of five major proteins: the nucleocapsid (N) protein, the viral transcriptase (L), the nonstructural (NS) protein, the matrix or membrane (M) protein, and the glycoprotein (G) (98, 139, 140). Other minor proteins have been detected by polyacrylamide gel electrophoresis but these proteins are not consistently found in purified VSV (9, 66, 138).

The nucleocapsid of the VSV virion is composed of the viral RNA and three proteins: N, L, and NS proteins. The N protein, which is tightly bound to the nucleic acid, has a molecular weight of 52,000 daltons, and constitutes 30% of the virion protein (10, 19, 21). Although no enzymatic activity has been found to be associated with the N protein, the estimated 1,100 to 2,300 N protein molecules per virion provide a significant amount of stability and resistance of the viral RNA to various degradative enzymes (9, 10, 19). The L protein, also associated with the nucleocapsid complex,

appears to be the viral transcriptase (6, 33, 34). This protein has a molecular weight of 190,000 daltons (L=large) and since there only are approximately 60 copies per virion, it constitutes only 2.5% of the total VSV virion protein (9, 10, 34). The third protein that is found associated with the VSV nucleocapsid is the NS protein which has a molecular weight of 30,000 to 35,000 daltons (103, 118). At one time it was considered to be a nonstructural protein since it is synthesized in relatively large quantities in the infected host cell, although only small amounts could be detected in the virion (67, 141). However, recent studies have shown the NS protein to be necessary for the L-protein (transcriptase) to function (34, 63). The possibility that the NS protein may modulate transcriptase activity has been emphasized by the observations that this minor component (2.3% of the virion protein) can exist in either a phosphorylated or a nonphosphorylated state (10, 95, 130).

The viral envelope, which surrounds the nucleocapsid of the virion, contains a lipid bilayer and the two remaining viral proteins: the glycoprotein (G: 69,000 MW) and the membrane or matrix protein (M: 29,000 MW) (19, 137). The G and M proteins compose 35% and 29%, respectively, of the total viral protein (10). The protein matrix on the intracellular side of the envelope is predominantly composed of the M protein (32). The G protein, the only glycoprotein of the VSV virion, is found inserted within the lipid bilayer and can be visualized as a peplomer or spike protruding from the surface of the virions with approximately 500 of these proteins per virion (19, 52, 85). Further evidence, which will be discussed later, indicates the G protein is the viral component that initially interacts with the host cell surface receptor. Although the VSV RNA and proteins are specified by the viral genome, the lipids in the envelope are derived from the host cell (86).

VSV possesses a very extensive host range, as reviewed by Bishop and Smith (10). Those cells which can be infected by VSV must share some common or similar receptor for the virus to attach and propagate. Common cell lines used to study VSV have been L-cells, baby hamster kidney cells and HeLa cells. Even cells of drosophila and mosquitoes can support the replication of VSV (124). Only a few cell lines, such as non-stimulated T-cells and domestic rabbit kidney and cornea cells, will not allow VSV to undergo a productive infection (25, 104, 133).

The infection and maturation process of VSV is similar to other enveloped viruses. The initial stage of infection involves the attachment of the virion to the host cell surface (10). There is evidence for entry of the VSV virion both by fusion of the viral membrane envelope with the cell surface membrane (44) and by phagocytosis of the intact virus particle (126). Although, biochemical evidence supports the mode of entry associated with membrane fusion, evidence is still lacking as to whether one mode of entry is particularly advantageous than the other (45).

Following the entry of the intact virion or the nucleocapsid alone, the viral components are synthesized with the aid of the protein synthetic machinery of the host cell. Because the RNA genome is antipolar and is incapable of acting as mRNA, transcription is a required step in the initiation of viral replication. Four different size classes of mRNA (31S, 17S, 14.5S, and 12S), that are transcribed from the viral genome, have been isolated (60, 93, 94, 99). In vitro translation of the purified mRNA species has demonstrated the 31S mRNA codes for the L protein (91), the 17S mRNA codes for the G protein, the 14.5S codes for the N protein (13, 74), and the 12S mRNA codes for both the NS and M proteins (13, 74, 116). The mechanism of transcription and post-transcriptional processing of these mRNAs has yet to be totally resolved. Current models propose that the

RNA polymerase initiates transcription at the 3' terminus of the RNA template genome and continuously moves toward the 5' end of the genome. A "processing enzyme" (of unknown origin) would then recognize specific sequences in the transcribed mRNA and cleave it into cistron-sized mRNAs. These cleaved mRNAs would then be polyadenylated at the 3' end and capped and methylated at the 5' end (7). Some studies have presented evidence that would contradict this proposed model. Hefti and Bishop (43) detected a small number of mRNAs which have a specific unblocked sequence at the 5' end which could be a precursor for the capped structures eventually isolated. Thus, in this situation, each mRNA may be independently initiated. To date, the actual mechanism of transcription of VSV mRNAs is not definitely known.

The process of replicating the negative strands of RNA is also not yet known. A complementary strand (the positive strand) of full length must be synthesized from a full length negative strand. Such a replicative intermediate has been detected in infected cells but not in in vitro RNA transcriptase assays (92). Since only monocistronic mRNAs, coding for individual virus structural proteins were transcribed in vitro, there must be either an alternate pathway for the synthesis of the positive and complementary strand by the virion-associated polymerase, or a replicase activity different from the RNA polymerase that is active in the in vitro synthesis system (7).

While viral transcription is occurring in the infected host cell, cellular metabolism is altered drastically by the presence of VSV resulting in a suppression of macromolecular synthesis including proteins and RNA (98, 105, 141). Huang et al. (60), have demonstrated the breakdown of host polyribosomes during the third hour of infection and the reformation of a different population of smaller and virus-specific polysomes during the fourth hour. Using these virus-specific polysomes investigators have shown

that the viral proteins N, NS, L, and M are synthesized with no evidence of post translation cleavage (73, 98, 141). In contrast to the other viral polypeptides, the G protein has a relatively complex pattern of synthesis and maturation. The G polypeptide is initially synthesized on polysomes associated with the rough endoplasmic reticulum and is directly inserted into the lipid bilayer where it is partially glycosylated by host glycosyl transferases (13, 41, 90, 136). The immature glycoprotein then migrates to the smooth endoplasmic reticulum of the Golgi apparatus where further glycosylation occurs just prior to the insertion of the G protein into the surface membrane (73).

The maturation and insertion of the G protein in the infected host cell surface appears to play an important role in viral maturation and release. Only after the G protein is inserted into the host membrane, can the M protein bind stably to the intracellular side of the surface membrane (73). If the G protein is not already inserted in the cell surface, the M protein is found in a soluble fraction, not membrane associated, and viral maturation is aborted (73). If the G protein is inserted into the cell surface, the VSV nucleocapsid with the RNA, N, L, and NS proteins, which have accumulated in the cytoplasm, will then assemble at the modified host membrane. After the assembly at the cell surface, the virus particle will be released by budding from the plasma membrane thereby acquiring an envelope that includes the host cell lipid bilayer (53).

During the infection of cells with VSV, two types of particles are most often produced: the infectious B-type particles and the defective-interfering T-type particles. The B-type particles are complete in all viral components, have the typical bullet-shpae morphology, and are infectious (137). The T-type particles are identical to the B-type particle in protein composition, however, T-type particles contain only a fraction of

of the RNA which results in a particle with a spherical morphology (61, 100). The T-type particles are defective because their RNA genome is incomplete and, therefore, can only replicate in host cells infected with B-type particles (119, 120, 131). Although the T-type particles are unable to replicate alone, they have the ability to interfere with VSV replication when present in the host cell at high concentrations (58). The mechanism by which T-type particles interfere with VSV replication is not definitely known but data suggests the T-type particles may interfere with viral RNA replication. The RNA from the defective particles appear to actively compete with the RNA from B-type particles for the limited number of the viral-specific RNA polymerase molecules thus reducing the number of complete RNA genomes and increasing the relative proportion of T-type particles (59).

Chemical Composition and Function of the Glycoprotein of VSV

The chemical and physical structure of the glycoprotein (G) of VSV has been studied by several investigators. Biochemical studies of the G protein have been aided by various isolation techniques which readily separate the G protein from the other viral proteins (9, 42, 70, 117, 132). Two oligosaccharide chains per glycoprotein molecule have been detected by several groups (35, 37, 117), although different numbers of oligosaccharide chains have been reported (96). Each oligosaccharide chain has a molecular weight of 3000 - 3400 daltons which represents approximately 5% of the glycoprotein by weight (35, 37, 96, 117). These oligosaccharides have been shown to be complex structures containing fucose, mannose, galactose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid (35, 36, 87). The carbohydrate moiety has been shown to be linked to the amide nitrogen of asparagine in the peptide portion of the G protein (114). The VSV glycoprotein contains a core structure which is similar to and common to other known asparagine-linked

oligosaccharides of other glycoproteins such as those isolated from chicken egg albumin or α-amylase from Aspergillus oryzae (77). The terminal sugar of the carbohydrate chain is neuramic acid and can be easily removed by treating the virion with neuraminidase (122, 123).

Investigators who have studied the carbohydrate composition of the G protein have disagreed on the actual number of specific carbohydrate residues per glycoprotein molecule (35, 87, 114). The variance in the number of sugar residues may, however, be a reflection of the host's influence on glycosylation of the G protein molecule as the carbohydrate composition depended on the host cell lines. This may be reasonable since the virus itself does not contain glycosyl transferases and, therefore, must rely on the glycosyl transferases of the host to add the oligosaccharide chains associated with the peptide of the G protein (10). Etchison and Holland (36) compared the G protein of virions which had been grown in four different cell lines. Both qualitative and quantitative differences occurred in the carbohydrate composition of the G protein. Approaching the question differently, Bruge and Huang (16) showed that when two unrelated envelope viruses Sindbis virus and VSV when grown in the same cell line, the glycoproteins of each virus contain the same carbohydrate composition. They also went on to show the carbohydrate composition of the progeny virions was different from the sugar composition of the original viruses used to infect the cells. These findings indicate that the host cell can influence the glycosylation and final chemical composition of the G protein molecules.

The G protein of VSV is the constituent of the peplomers or spikes that can be seen protruding from the virion (22). When VSV virions are exposed to proteolytic enzymes such as trypsin, pronase, chymotrypsin, and bromelain, the G protein was selectively removed (17, 85). All that remains of the G protein after enzymatic digestion is a non-glycosylated hydrophobic

tail which is embedded in the virion envelope (97). Just recently, Katz and Lodish (69), presented evidence that the G protein is found in a transmembrane, asymetric orientation in the viral envelope with the carbohydrate moiety extending outward from the surface and a small portion being exposed on the cytoplasmic side of the envelope. Iodination of intact VSV virions by methods used to locate the surface proteins of cells and other viruses, e.g. chloramine T or lactoperoxidase procedures, disclosed that the G protein was the only viral protein significantly labeled and further substantiated the outer location of the G protein (89, 142).

During the infectious cycle of VSV the G protein functions as the viral component which initially interacts with, and attaches to, the plasma membrane of the susceptible cell. A loss of infectivity, often .0001% of the original titer, is seen with VSV virions that are treated with trypsin to remove the G protein (8). Temperature-sensitive mutants of VSV, belonging to complementation group V, which are defective at elevated temperatures in the synthesis or maturation of the G protein, are also incapable of infecting cells unless they are co-infected with another envelope virus like avian retroviruses (78). By comparing antiserum prepared against the VSV G protein and other structural proteins, it was shown that only antiserum against the G protein would neutralize viral infection (31, 70). All of these observations suggest that the G protein is the viral component that is involved in attachment or adsorption of the virion to the host cell.

The G protein has also been shown to be responsible for the ability of VSV to hemagglutinate goose erythrocytes (2). Hemagglutination of the red blood cells can be blocked by neutralizing antibody which is directed against the G protein (137). Also, isolated G protein is capable of hemagglutinating goose erythrocytes at a pH of 6 and at 4°C (50, 85). These hemagglutination studies also suggested that no neuraminidase was

found associated with VSV virions, which is contrary to what is found in influenza virus (72). When intact VSV virions are treated with V. cholera neuraminidase to remove the terminal neuramic acid of the G protein, a loss of 99% in VSV infectivity and a similar decline of hemagglutination activity are measured (122). VSV virions that were grown in A. albopictus mosquito cells were found to lack sialic acid and were not able to efficiently infect cells (124). Therefore, the presence of neuramic acid appears to be helpful to the ability of the virus to interact with the cell.

Little information is available concerning the cellular receptor for VSV attachment. One study where BHK-21 cells were treated with trypsin, neuraminidase or DEAE-dextran showed that attachment of intact VSV was increased (123). The enhancement in binding could be attributed to the enzymatic removal of proteins that interfere with viral attachment and/or increasing the relative density of the cell receptor which would allow multivalent binding of the virus. By using the isolated G protein as a probe, hopefully more information can be collected so this host-virus interaction can be better understood.

SECTION III

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LITERATURE CITED

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SECTION IV

MANUSCRIPT: ISOLATION OF THE GLYCOPROTEIN OF VESICULAR STOMATITIS VIRUS AND ITS BINDING TO CELL SURFACES

SUMMARY

The glycoprotein (G) of vesicular stomatitis virus was radiolabeled, extracted and purified to allow the study of its potential interaction with host cell surfaces. Incubation of BHK-21 cells with the radiolabeled viral glycoprotein resulted in a rapid attachment of the viral component to the cell surface. The attachment was shown to be temperature dependent and saturable in that approximately 3 X 10^5 molecules bound per cell. The deletion of Mg^{++} or Ca^{++} from the incubation medium had little affect on the glycoprotein binding. Treatment of the isolated G protein and intact virions with neuraminidase did not significantly decrease their binding to BHK-21 cells. Preincubation of cells with trypsin did not decrease the attachment of VSV virions nor the binding of purified G protein. Treatment of cells with phospholipase A or phospholipase C suggested that the binding of the glycoprotein and the intact virion may have been dissimilar. Unlabeled glycoprotein was shown to competitively inhibit binding of the labeled molecules although the presence of intact virions did not inhibit attachment of the G protein. Likewise, saturating amounts of the glycoprotein did not decrease binding of vesicular stomatitis virus to BHK-21 cells. These observations suggested that the isolated glycoprotein either bound to cell surface components that were distinct from the virion receptor or that the manner of the purified glycoprotein attachment was different than the G protein still associated with the intact virion. Chemical cross-linking and diagonal two-dimensional gel electrophoresis were used to identify and to compare the cell surface components responsible for glycoprotein and virion attachment.

INTRODUCTION

The initial interaction between viruses and the host cell surface has been the subject of numerous studies. In most cases, however, investigators have focused on the physical and chemical properties of the viral components that are responsible for virion attachment (Bose & Sagik, 1970; Lonberg-Holm & Korant, 1972; Philipson & Lindberg, 1974). Until recently most studies involving the host cell surface receptor complexes associated with virus attachment have been limited to indirect examination by enzymatic analyses and many have been restricted to erythrocyte surfaces. For instance, sialic acid of a surface glycoprotein of erythrocytes was shown to mediate influenza virus attachment (Kathan et al., 1961; Hoyle, 1968) and glycoproteins that contain sialic acid, present in serum and respiratory fluid, were shown to prevent virion attachment to susceptible cells (Howe & Lee, 1970). This host cell surface glycoprotein contains most of the N-acetylneuramic acid of the erythrocyte membrane (Cook et al., 1961; Dannon et al., 1965) and interestingly, also contains the determinants of the M and N blood group substances (Kathan et al., 1961; Howe et al., 1963).

A cell surface glycoprotein, devoid of sialic acid, has been isolated and characterized as being responsible for the binding of adenovirus, type 7, to susceptible cells (Neurath et al., 1970). More recently, a glycoprotein (gp71) of murine leukemia virus has been used as a molecular probe to identify the host cell component responsible for virion attachment. The binding properties and requirements of the gp71 molecule were shown to be similar to the intact virion (DeLarco & Todaro, 1976; Fowler et al., 1977). Further studies of the cell surface component have suggested that the murine leukemia virus binds to a lipoprotein (Kalyanaraman et al., 1978).

Vesicular stomatitis virus (VSV), a rhabdovirus that possesses an unusually broad host range which includes established cell lines of arthropod, avian, and mammalian species, has a sole glycoprotein (G) that is responsible for virion attachment (Kelley et al., 1972; Bishop et al., 1975). Antiserum prepared against the glycoprotein neutralizes viral infectivity while antibodies directed against other VSV structural proteins do not inhibit viral infection (Kelley et al., 1972; Dietzschold et al., 1974). Schloemer and Wagner (1975) found that the cellular receptor for VSV was resistant to treatment with neuraminidase and trypsin. Beyond this, little is known about the chemical nature of the cellular receptor for VSV or the nature of the viral interaction with the host cell surface. However, the characterization of the chemical and physical properties of the VSV-host cell interaction may be fundamental to our understanding of the reasons why this virus has such a broad host range. These studies were initiated to determine if the isolated G protein of VSV could bind to cell surfaces and if the nature of the G protein attachment was related to the interaction of the cell with the intact VSV virion.

METHODS

Cell culture. Baby hamster kidney cells (BHK-21), obtained from International Scientific Industries, Cary, Illinois, were maintained as monolayer cultures by routine cell culture methods (Rabinowitz et al., 1976). The BHK-21 cells were grown in Earle salts supplemented with 7% virus-screened fetal calf serum, 10% tryptose phosphate, 2mM glutamine, 1% non-essential amino acids, 1% minimal essential medium vitamins, and penicillin (1000 U/l), streptomycin (1 mg/l), fungizone (2.5 mg/l, and gentamycin (20 mg/l) (Grand Island Biological Co.). BHK-21 cells were grown to confluency in 32 oz. sterile glass bottles by incubation at 37°C in a 5% CO_2- 95% air atmosphere in a water-jacketed incubator.

Virus preparation and purification. The Indiana serotype of VSV was added to monolayers of BHK-21 cells at a multiplicity of infection (MOI) of 0.1 plaque forming units (PFU) per cell and allowed to adsorb for 30 min at 25°C. Culture media was added and whenever appropriate, 5 μCi/ml of ^{3}H-fucose and/or 5 μCi/ml of ^{35}S-methionine (New England Nuclear Corp.) were added. The infected cells were incubated at 37°C for 18-24 h. Mature virions released into the medium were isolated by first removing cellular debris by slow speed centrifugation (5,000 x g for 10 min). VSV virions were then pelleted from the resulting supernatant fluid by ultracentrifugation at 32,000 x g for 3 h at 4°C. The virus pellet was suspended in TEN buffer (50mM Tris-HCl, 100mM NaCl, 5mM EDTA, pH 7.4), briefly sonicated (Sonifier Cell Disruptor, Model W140D, at a setting of 3 for 5-10 sec), and repelleted at 136,000 x g for 1.5 h at 4°C. The final VSV pellet, containing both B and T-type particles (Haung et al., 1966), was resuspended in either TEN buffer or Hanks' balanced salt solution (HBSS) (Hanks & Wallace, 1949), briefly sonicated, and stored at -90°C.

Plaque and plaque reduction assays. Ten-fold serial dilutions of purified VSV were prepared in HBSS and 0.1 ml was plated onto monolayers of BHK-21 cells cultured in six-well plates (35 X 10 mm, Linbro, Co.). After virus was allowed to adsorb to cells for 30 min at 25°C, the monolayers were overlayed with growth media supplemented with 0.37% agar (Difco Laboratories) as a solidifying agent. All samples were plaqued in duplicate and plates were incubated at 37°C for 24-48 h. After incubation, the agar medium was removed and the plates were stained with crystal violet (Hughes et al., 1979).

Isolation and purification of G protein. The G protein was isolated by a modification of a technique used by Hale et al. (1978). Nonidet P40 ((NP40), Shell Oil Co.) was added to purified virions to a final concentration of 1% (V/V) for 20 min at 25°C with occasional stirring. The nonsolubilized nucleocapsids were then separated from the solubilized envelope G and M proteins, by pelleting the nucleocapsids through 20% sucrose at 136,000 x g for 90 min at 4°C. The viral envelope proteins, which remained above the 20% sucrose, were collected for analysis by SDS-PAGE and further purification. The M and G protein were then separated on a Sephadex G-75 column (56 X 1.1 cm) using phosphate buffered saline ((PBS) 0.4M NaCl, 2.7mM KCl, 6.7mM Na_2HPO_4, 1.4mM KH_2PO_4, pH 7.4) to elute the two proteins. The G protein eluted in the void volume, while the M protein eluted in later fractions. The fractions containing only the G protein were pooled and used to measure binding of the VSV glycoprotein to BHK-21 cells.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Viral proteins of VSV and various protein samples were analyzed by SDS-PAGE according to the method described by Laemmli (1970). The sample proteins were electrophoresed (2.5mA/gel) through a 3% polyacrylamide stacking gel (2 cm) and a 9% polyacrylamide separating gel (10 cm). With radioactive samples, the gels were frozen following electrophoresis and sliced into 1mm slices. The

slices were mixed overnight in a NCS: scintillation cocktail mixture (Hughes et al., 1979) and the radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer, model 3320. Those gels which contained unlabeled protein were stained following electrophoresis with Coomassie brilliant blue and were scanned at 500nm using a Gilford spectrophotometer (Model 250) with a linear transport module. Areas under viral protein peaks were integrated using a Numonics electronic graphic calculator. In all instances of gel electrophoresis, samples of bovine serum albumin (MW 68,000 daltons), ovalbumin (MW 45,000 daltons), and chymotrypsinogen (MW 25,000 daltons) were co-electrophoresed as molecular weight standards to determine the molecular weights and identity of the viral proteins.

Antiserum to G protein. Isolated G protein, approximately 0.5-1.0 mg, was added to complete Freund's adjuvant and injected subcutaneously into New Zealand white rabbits. A booster injection containing incomplete Freund's adjuvant was given every two weeks following the initial injection. Serum was collected every two weeks and tested for the ability of the antiserum to neutralize viral infection by plaque reduction assay. Serum with high neutralizing titers were pooled and IgG immunoglobulins were purified as previously described (Heide & Schwick, 1978; Fahey & Terry, 1978). Briefly, the serum was precipitated repeatedly with sodium sulfate, the precipitate was dissolved in 0.03M Tris-HCl-phosphate buffer (TP), pH 8.0 and was then fractionated on a DEAE-cellulose column (80 X 2.5 cm) previously equilibrated with the TP buffer. The IgG which elutes in the void volume was then concentrated in an Amicon ultrafiltration cell (Amicon Corp.) using a type PM-10 ultrafiltration membrane. In order to reduce non-specific reactions, purified antiserum (0.1 mg) was absorbed for 30 min at 37°C with 1 X 10^7 BHK-21 cells

prior to use. Neutralization titers of antiserum were determined by a plaque reduction assay. Five-fold dilutions of antiserum were reacted with an equal volume of VSV (1×10^8 PFU) for 30 min at 37°C and then plaqued on BHK-21 monolayers as described above.

Binding of G protein to BHK-21 cells. ^{3}H-fucose and ^{35}S-methionine-labeled G protein, at the designated concentrations, was mixed with two volumes of binding solution (28mM NaCl, 9mM glucose, 7.6mM KCl, 1.4mM $MgSO_4.H_2O$, 3.4mM KH_2PO_4, pH 7.4) and one-third volume of 12.6mM $CaCl_2$. The BHK-21 cells were prepared for the binding studies by scraping the cell monolayers with a rubber policeman, pelleting the cells by slow speed centrifugation and then suspending them in HBSS. The labeled G protein was added to 7.5×10^6 - 1×10^7 cells for the binding assay. Following incubation, the cells were pelleted and the supernatant discarded. Cells were washed twice with HBSS and the final pellet was solubilized with 1N NaOH. The solubilized cell solution was dissolved in 3.5 ml of Biofluor scintillation cocktail (New England Nuclear, Corp.) and 2 drops of 1N acetic acid were added to each vial and the radioactive incorporation determined in a Packard Scintillation spectrometer. The number of G protein molecules which bound per cell was calculated from the specific radioactivity of the G protein after a zero time point as background was subtracted. The specific radioactivity of the G protein preparation isolated from labeled VSV virions ranged from 4.3 to 8.7×10^5 CPM/mg protein. Protein concentrations were determined by procedure of Lowry et al. (1951) using bovine serum albumin as a protein standard.

Enzymatic treatment of BHK-21 cells. Ten ml of a trypsin solution (1:250) or 1 U of phospholipase C (Sigma Chem. Co.) were incubated with 7.5×10^6 BHK-21 cells for 30 min at 37°C. Five units of phospholipase A (Sigma Chem. Co.) were added to 7.5×10^6 BHK-21 cells for 30 min at 25°C.

After enzymatic treatment, cells were pelleted and washed 3 times with HBSS prior to being used in a binding assay. Phospholipase C was suspended in 0.5mM phosphate buffer, pH 7.4 and phospholipase A was suspended in 0.22M NaCl, 20mM $CaCl_2$, and 1mM EDTA, pH 7.5.

Neuraminidase treatment of virus and G protein. Approximately 5U of V. cholera neuraminidase (Cal Biochem-Behring Corp.) was incubated with 1 X 10^7 PFU of ^{35}S-methionine-labeled VSV for 90 min at 37°C. Treated virions were pelleted by ultracentrifugation, suspended in HBSS, and infectivity was analyzed by plaque assay on BHK-21 cell monolayers. Approximately 8 g of labeled G protein was treated with 0.5U of neuraminidase linked to agarose beads (Sigma Chem. Co.) at 37°C for 3 hours and the enzyme beads were then removed by centrifugation. Attachment of enzyme treated G protein and virions was evaluated and compared to G protein or virions treated in the same manner but without neuraminidase.

Crosslinking G protein and VSV to cell surfaces. Saturating amounts of labeled G protein (50 µg) or intact virions (MOI=100) were adsorbed to 6 X 10^7 BHK-21 cells for 30 min. Cells were washed and 6.0 mg of the chemical crosslinker, dimethyl-3,3'-dithiobispropionimidate dihydrochloride (DTBP, Pierce Chemical Co.), was added to cells for 30 min at 4°C according to the procedure outlined by Takemoto et al. (1978). After incubation with the crosslinker, cell membranes were isolated by treating crosslinked cells with 1% NP40 for 15 min on ice, removing nuclei by centrifugation and then diluting the supernatant, containing the solubilized cell membranes, two-fold with the final buffer of Takemoto et al. (1978). A sufficient quantity of purified IgG (0.2 mg) directed against the G protein and preabsorbed with BHK-21 cells was incubated with the cell membrane fraction for 30 min at 37°C and then placed at 4°C overnight. The resulting precipitate was pelleted by centrifugation, suspended in sample buffer without

mercaptoethanol (Kelly & Luttges, 1975) and labeled by the chloramine T iodination method (Moore et al., 1974). As a control, cells which were not exposed to either G protein or VSV virions were crosslinked with DTBP, cell membrane fractions were collected, reacted with antiserum and iodinated in a similar manner.

Crosslinked species precipitated by the IgG were resolved by diagonal two dimensional electrophoresis. Approximately 4 X 10^6 CPM of each sample were electrophoresed (10mA/gel) through a 3% acrylamide stacking gel and 9% acrylamide separating gel in the absence of 2-mercaptoethanol. After electrophoresis, sample tracks were cut out, placed horizontally above another prepared polyacrylamide gel, and immobilized on the top of the slab gel with 0.5% agarose, .38 M Tris-HCl (pH 8.8), and 0.5M mercaptoethanol to cleave the crosslinked species and to allow their separation by electrophoresis in the second dimension. Gels were initially electrophoresed at 10mA/gel until the tracking dye approached the separating gel after which electrophoresis was continued at 30mA/gel. After electrophoresis, gels were stained and fixed with 0.25% Coomassie blue in 50% methanol and 5% acetic acid, dried by vacuum, and exposed to Kodak XRP-1 film for 1-2 days.

RESULTS

Isolation and purification of the G protein

Initial studies were carried out to determine the protein and glycoprotein composition of the purified VSV virions and the efficiency of NP40 in separating the virion envelope and nucleocapsid components. SDS-PAGE analysis of complete VSV virions clearly separated the major structural proteins; and virus previously labeled with ^{3}H-fucose, which labels only the viral glycoprotein (G), provided a convenient marker (Fig. 1). The relative proportions of each protein in the intact virions and the nucleocapsid and soluble membrane fractions following NP40 extraction were determined by staining the gels with Coomassie brilliant blue, integrating the areas under each viral peak and comparing the area to the total. The protein composition of the virion was 3.2% L protein, 28.5% G protein, 33.7% N protein, and 34.1% M protein (Table 1). The NS protein, which mormally constitutes only 1% to 2% of the viral protein (Bishop & Smith, 1977), could not be accurately quantitated because of the small amount and the fact that it often migrates close to the N protein.

NP40 extraction provided an efficient method to separate the nucleocapsid and envelope proteins of VSV. The nucleocapsid pellet contained 100% and 96% of the L protein and N protein, respectively. Approximately 75% of the G protein was solubilized by treatment with NP40 and SDS-PAGE analysis of this soluble fraction showed that only a trace of the major nucleocapsid protein (N) was present (Table 1). The G and M proteins of the virion envelope were separated by gel filtration on a Sephadex G-75 column as described in the Methods section. SDS-PAGE analysis of the G protein fraction, which eluted in the void volume, demonstrated that the preparation was composed of over 97% G protein and contained less than 3% M and N (fig. 2).

The purified G protein appeared to be intact since its migration by SDS-PAGE was identical to the glycoprotein of intact virions. This purification procedure allowed a recovery of 55% of the initial G protein of the VSV.

Binding of G protein to BHK-21 cells

In preliminary experiments we found that the purified radiolabeled G protein of VSV bound tightly to BHK-21 cells. In order to optimize the binding of the G protein to BHK-21 cells, we measured the effects of varying the concentration of G protein, incubation temperature, time of incubation, the composition of the binding medium, and cell concentration. When increasing concentrations of G protein were incubated with 1 X 10^7 BHK-21 cells at 25°C, the amount of G protein bound was essentially maximal at 5.0 µg (Fig. 3). Increasing the concentration of G protein to over 15 µg per reaction resulted in little additional binding. These kinetics suggested that the binding reaction was saturable and that there were a definitive number of average binding sites per cell ($\sim$ 2-3 X 10^5).

The kinetics of G protein binding to BHK-21 cells was shown to be relatively rapid in that 57% and 78% of the binding reaction was completed within 5 min of incubation at 4°C and 25°C, respectively (Fig. 4). The remaining G protein that bound during the subsequent 10 min of incubation appeared to occur at a different, and slower rate. Incubation of G protein and BHK-21 cells for periods longer than 15 min at 4°C and 25°C did not result in increased binding. A greater amount of G protein bound at 37°C although this may have been the result, in part, of an internalization of glycoprotein molecules. In order to minimize this possibility, subsequent experiments were carried out at 4°C or 25°C.

Several experiments were carried out to assess the possible role of Mg^{2+} and Ca^{2+} in the binding of G protein to BHK-21 cells. However, increasing

the Mg^{2+} or Ca^{2+} concentration by 2.5-fold had no measurable effect on G protein binding and the deletion of these ions from the incubation medium did not decrease glycoprotein binding (data not shown).

Specificity of G protein binding

The saturable kinetics of G protein binding suggested that the interaction of the glycoprotein with the cell surface involved a limited number of sites that may be specific (Figs. 3 and 4). If this was true the binding of G protein should have been dependent on cell concentration and an excess of unlabeled G protein should have competed with the radiolabeled glycoprotein. Various concentrations of BHK-21 cells were incubated with a saturating amount of radiolabeled G protein (Table 2). A total of 3.1-3.5 X 10^5 molecules of G protein bound per cell regardless of the number of cells used. Under similar incubation conditions calculations from 34 independent determinations throughout this study showed that 3.3 X 10^5 G protein molecules bound per BHK-21 cell.

Further evidence for a saturable binding of G protein was obtained by mixing known concentrations of unlabeled G protein with a constant amount of radiolabeled G protein. The binding of 0.6 µg of added labeled G protein was inhibited by unlabeled G protein in a competitive fashion in that the measured decrease in labeled G protein bound was consistent with that calculated on the basis of competitive inhibition (Table 3). However, the calculated and observed G protein binding was only consistent when 1.4 µg and 2.8 µg of unlabeled G protein was present in the reaction mixtures. When 0.8 µg of unlabeled G protein was mixed with 0.6 µg of the radiolabeled glycoprotein, 61% of the labeled protein bound which was well above the 42% calculated. This indicated that 0.6 µg of G protein was not sufficient to saturate all cell binding sites and that at least 2.0 µg of G protein was

sufficient to provide saturation kinetics with 1 X 10^7 cells. This observation was consistent with the saturation curve illustrated in Fig. 3. In addition, this observation eliminated the possibility that the measured number of G protein molecules bound per cell was appreciably a result of G protein aggregation.

Comparison of G protein and VSV binding

Now convinced that the binding of G protein was saturable and involved approximately 3 X 10^5 sites per cell, we felt that comparisons between the binding of purified G protein and VSV virions could be made with confidence. Since Schloemer and Wagner (1975) reported that the infectivity of VSV could be decreased by the treatment of virions with neuraminidase, we incubated both the virions and the G protein with neuraminidase as described in the Methods and measured their subsequent ability to bind to BHK-21 cells. Neuraminidase treatment resulted in a 1.5 to 2.0 log loss of infectious VSV, as measured by plaque assay, although virion attachment was reduced by only 25% (data not shown). Treatment of the G protein with neuraminidase also had little affect on the ability of the isolated glycoprotein to bind to cell surfaces. Although antibody, directed against the G protein of VSV, clearly neutralizes viral infectivity (Kelley et al., 1972; Dietzschold et al., 1974), it is not known if the antiserum directly inhibits virion attachment or interferes with later stages of infection. We found that incubation of VSV or G protein with anti-G IgG sufficient to reduce viral infectivity by four logs did not decrease either virion or the purified protein binding to BHK-21 cells (data not shown). These results, therefore, had little value in discriminating between the attachment properties of the intact VSV virions and the purified G protein.

The ability of BHK-21 cells to bind intact VSV virions and isolated G protein was not diminished by preincubation of the cells with trypsin (Table 4). However, phospholipase A increased their ability to bind G protein while the attachment of intact VSV virions was somewhat reduced. The enhanced binding of G protein by pre-exposure of the cells to phospholipase A was most likely a reflection of an increased exposure of cell surface receptors to the glycoprotein. Treatment of BHK-21 cells with phospholipase C slightly reduced G protein binding although the enzyme had little affect on the attachment of intact VSV (Table 4). The differences in cellular binding that resulted from these enzymatic treatments were not of a magnitude to be unequivocal. Although this was the first evidence that the nature of the binding of the isolated G protein and the VSV virions was different, a more direct assessment was necessary.

Competitive binding by the virions and the G protein

Since the binding of radiolabeled G protein was inhibited by unlabeled G protein (Table 3), a more direct approach was available to compare the attachment of the glycoprotein and the VSV virions. The presence of a saturating quantity of G protein did not interfere with the binding of radiolabeled VSV virions to BHK-21 cells (Table 5). In addition, the presence of intact virions did not reduce the amount of radiolabeled G protein that was bound (Table 5). These observations clearly indicated that the purified glycoprotein, although binding to a saturatable cell surface component, did not bind in a fashion that was identical to the intact virion. In support of this conclusion, we also could not find evidence that the G protein, bound to BHK-21 cells prior to the addition of VSV, was displaced when virions were added.

Chemical crosslinking of the G protein to BHK-21 cells

In order to compare the cell surface components that primarily bound the isolated G protein and the G protein in the intact virion, we crosslinked the bound molecules with DTBP, an imidoester that reacts with primary amines and is cleaved by mercaptoethanol. The G protein or intact VSV virion was first bound to BHK-21 cells, reacted with DTBP, and the membranes were then solubilized with NP40 as described in the Methods. The crosslinked G protein-surface membrane complexes were precipitated with anti-G IgG and proteins were iodinated with ^{125}I. When radiolabeled G protein were used to measure total recovery by this method, over 65% of the initial G protein was recovered by precipitation with the antibody. The iodinated components were finally resolved by two dimensional diagonal electrophoresis to allow the identification of cell surface moieties that were associated with the free and virion bound G protein.

In the absence of both G protein and the DTBP crosslinker, only two cell surface components were found to be linked by disulfide bonds (Fig. 5a, arrows). Based on their positions in the slab gel and their migration in the 1st dimension (the crosslinked form), these cell surface components appeared to naturally exist as homodimers and homotetramers. In the presence of the DTBP crosslinker, but the absence of either isolated G protein or VSV virions, a total of six host cell membrane components were found to migrate at positions out of the diagonal (Fig. 5a). When G protein was bound to the cells prior to crosslinking with DTBP, ten additional host cell proteins were found to be crosslinked (Fig. 5b). Of these, only components 1 (79,000 daltons) and 2 (26,000 daltons) appeared to be crosslinked with the G protein. According to their migration in the 1st dimension as crosslinked species, the G protein could have been complexed to both cell components. For instance, cell surface component 1, crosslinked with the

G protein, could migrate at 137,000 daltons rather than the accumulative 148,000 daltons, because the G protein itself contains an internal disulfide bond and will migrate faster in the absence of mercaptoethanol (Mudd & Swanson, 1978). In a similar fashion, a crosslinked complex of three cell components 2 and a G protein would migrate at approximately 137,000 daltons in the 1st dimension.

Although not covalently crosslinked to the G protein by DTBP, cell components 3, 4, 5 and 6 were crosslinked as a result of the addition of the glycoprotein (Fig. 5b). Most likely these cell components were originally complexed with the G protein, and precipitated by the anti-G IgG, although amine groups of the G protein and these cell components were not situated properly for DTBP to form a crosslink. This would escape direct analysis since the presence of SDS would dissociate the G protein from the chemically crosslinked cell components. These four crosslinked species were not visualized when intact virions were reacted with the cell surface (Fig. 5c).

In addition to the above four cell components that were crosslinked when G proteins, but not VSV virions, were added, other differences were clearly established that explained the inability of the isolated G proteins and the virions to compete in surface binding. For instance, only cellular component 2 (26,000 daltons) and not component 1 was crosslinked to the G protein when intact virions were bound to the cell surface (Fig. 5c). In addition, cell components 7 and 8, as well as 9 and 10, were crosslinked when intact virions were used. None of these four cell proteins were crosslinked when isolated G protein was bound (Fig. 5b).

DISCUSSION

When VSV virions were solubilized with Np40, the two envelope proteins, M and G, were found in the soluble fraction. A single separation step using Sephadex G-75 allowed the recovery of 55% of the virion G protein in a fraction that was 97% pure. Although Triton X-100 (Kelley et al., 1972) was also used, we did not find that it was as efficient as NP40. The purified G protein appeared to be intact and to migrate on SDS-PAGE gels identical to the original virion glycoprotein. The G protein bound efficiently to BHK-21 cells, at a rapid rate and in a saturable manner, resulting in 3 X 10^5 molecules bound per cell (Table 2). This was similar to previous observations involving isolated proteins of murine leukemia virus (DeLarco & Todaro, 1976), and adenovirus (Philipson et al., 1968). This is in contrast to the unsaturable kinetics measured with myxoviruses (Lonberg-Holm & Philipson, 1974). The fact that unlabeled G protein competed with the binding of labeled molecules suggested that there are a definite number of receptor sites on the cell surface and that the attachment kinetics did not reflect an aggregation of the glycoprotein molecules (Table 3). This is similar to the report of Weissman et al. (1977) who showed that the binding of the murine leukemia virus was saturable and not a result of aggregation. Although the binding of the VSV G protein was more rapid at 25°C than at 4°C, the total number of molecules bound at 15 min was very similar (Fig. 4). Longer incubation periods did not lead to more binding. Increasing concentrations of either Mg^{++} or Ca^{++}, or their deletion from the incubation medium, had no affect on G protein binding to BHK-21 cells. This is in contrast to the cation sensitivity of binding of the gp71 of murine leukemia virus to isolated membranes of 3T3 cells (Kalyanaraman et al., 1978).

Several experiments were carried out to compare the properties of the binding of the VSV G protein and the intact virion. Treatment of the VSV glycoprotein with neuraminidase did not reduce its ability to attach to BHK-21 cells. Although neuraminidase reduced virion infectivity by approximately 99%, the ability of the virions to attach was only decreased by 25%. Preincubation of the BHK-21 cells with trypsin did not affect the binding of the purified G protein or the intact virions. A similar observation has been reported by Schloemer and Wagner (1975) where trypsinization of BHK-21 cells actually increased the ability of VSV at attach. Treatment of cells with phospholipase A and phospholipase C, suggested that the cell surface components responsible for the binding of the G protein and the virions may have been different (Table 4). However, the differences were small compared to the observations of Kalyanaraman et al. (1978) and, therefore, difficult to interpret. However, the inability of the G protein to compete with the binding of the intact virion indicated that there were independent attachment sites for the glycoprotein and the virion (Table 5). In a similar fashion, the virion was unable to compete against the binding of the G protein. Even preadsorption of either the G protein or the VSV virion did not affect the subsequent binding of the other. No evidence could be found that the relative affinity of the virion for the cell surface receptor was greater than that of the purified G protein.

A direct examination of the cell surface moieties responsible for the binding of the G protein and the intact virion involved the use of a chemical crosslinker and a purified anti-G IgG preparation. In both cases the presence of the ligand caused the crosslinking of several cell surface components (Fig. 5). There were some surface proteins that appeared to be associated with the binding of both the purified G protein and the intact virions. More importantly, and consistent with their inability to

cross-compete for binding, the majority of cell surface components associated with the attachment of the G protein and the intact virion were not shared. Evidently, the manner in which the G protein is presented, when freed from the virion envelope, is quite different than the closely packed G protein molecules at the virion surface. It is possible that the clusters of G protein in the virion envelope may have a different selectivity for all surface receptors than the single G protein molecules. Perhaps the insertion of the G protein into liposomes would provide a cell surface probe that would be more similar to the intact virion. In any event, it is clear from our data that isolated glycoproteins known to be responsible for viral attachment, must be used with caution as molecular probes for the identification of surface receptors associated with viral attachment. This may be particularly important with viruses like VSV that have a very broad host range and, perhaps, can attach to a wide spectrum of host cell surface components. Perhaps this experimental approach is most appropriate for viruses that have a highly restricted host range and, therefore, interact with a limited number of cell surface components (Neurath et al., 1970; DeLarco & Todaro, 1976; Kalyanaraman et al., 1978).

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Fig. 1. Separation of viral proteins by SDS-PAGE. VSV was isolated from infected BHK-21 cells and isolated as described in the text. The viral proteins were then separated by electrophoresis on an SDS-polyacrylamide gel, stained with Coomassie blue, destained with 10% acetic acid, and scanned at 550nm using a Gilford linear transport scanner (solid line). In a separate experiment, ^{3}H-fucose was added to the infected BHK-21 cells in order to radioactively label newly-synthesized VSV. Following virus isolation and electrophoresis, the polyacrylamide gel was sliced into 1 mm slices. The slices were dissolved in an NCS solution and the radioactivity (broken line) in each slice was determined as described in the text.

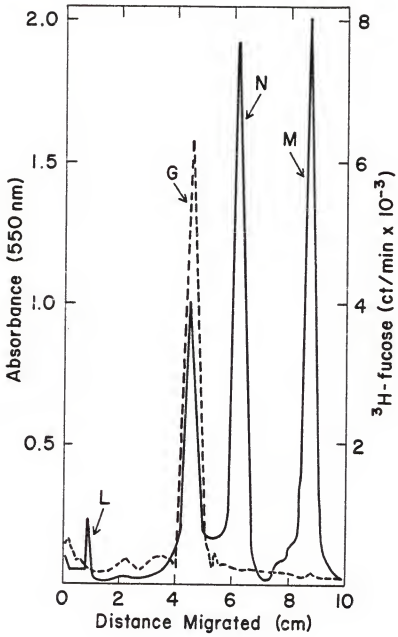
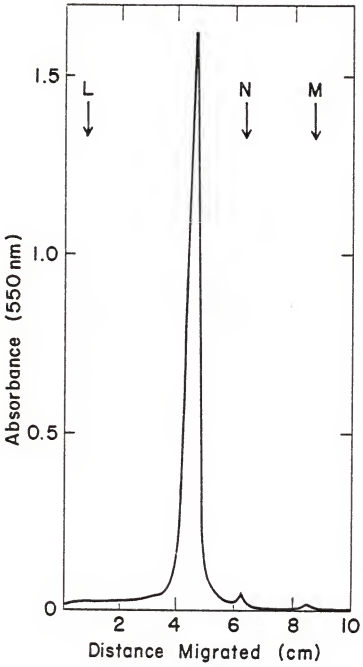
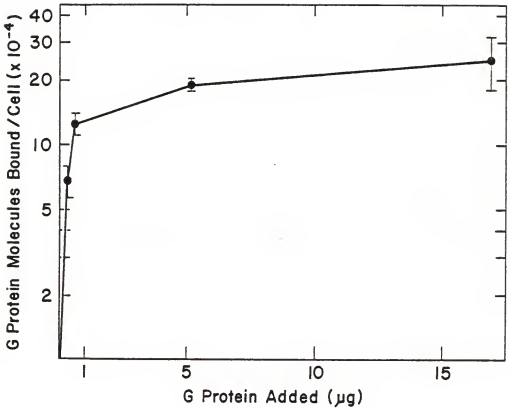
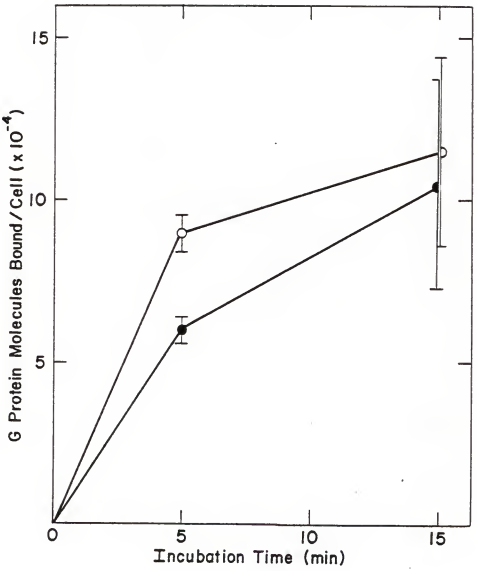

2.0
1.5
1.0
0.5
Absorbance (550 nm)
8
6
4
2
^{3}H-fucose (ct/min x 10^{-3})
L
G
N
M
0
2
4
6
8
10
Distance Migrated (cm)

Fig. 2. Analysis of isolated G protein by SDS-PAGE. The M and G envelope proteins, which were solubilized by extraction with NP40, were separated by Sephadex G-75 gel filtration. The void volume fraction, which appeared to contain almost solely the G glycoprotein, were separated by gel electrophoresis, stained with Coomassie blue and scanned at 550nm. The M protein was detected in fractions eluting after the void volume (data not shown).


1.5
1.0
0.5
0
L
N
M
Absorbance (550 nm)
2
4
6
8
10
Distance Migrated (cm)


G Protein Molecules Bound / Cell (x 10⁻⁴)
40
30
20
10
5
2
1
5
10
15
G Protein Added (μg)


15
10
5
0
5
10
15
G Protein Molecules Bound/Cell (x 10⁻⁴)
Incubation Time (min)

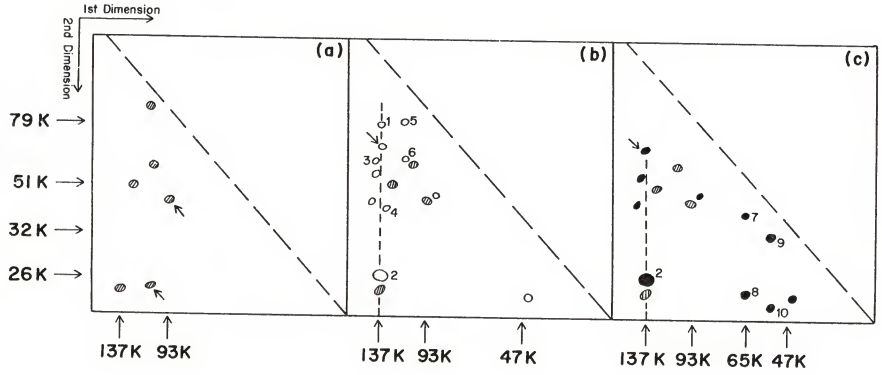

1st Dimension
2nd Dimension
79 K
51 K
32 K
26 K
(a)
(b)
(c)
137K 93K
137K 93K 47K
137K 93K 65K 47K

Table 1. FRACTIONATION OF THE VSV PROTEINS

Viral protein	% of protein in intact virions*	% present in Nucleocapsid pellet†	% present in Soluble fraction†
L	3.2 ± 0.5	100.0 ± 0.0	0
N	33.7 ± 1.8	96.4 ± 1.2	3.6 ± 1.2
G	28.5 ± 1.5	25.3 ± 2.8	74.7 ± 2.9
M	34.6 ± 1.3	43.8 ± 5.3	56.2 ± 5.2

* The VSV proteins were separated by SDS-PAGE, stained with Coomassie blue, and the gels were scanned at 550nm as in Fig.1. The area under each protein peak was integrated to determine the % each VSV protein was of the total virion protein content.

† The VSV virions were dissociated by NP40 treatment, followed by centrifugation into a nucleocapsid pellet and soluble fraction as described in the text. The % of each protein present in each fraction was determined from SDS-PAGE analysis of the fractions. All values represent the mean of 5 to 8 independent experiments ± S.E.M.

Table 2. EFFECT OF CELL NUMBER ON G PROTEIN BINDING*

Total BHK-21 cells present ($\times 10^6$)	Total number of G molecules bound ($\times 10^{11}$)	G protein molecules bound per cell ($\times 10^5$)
10.0	31.4	3.1 ± 0.2
7.5	26.6	3.5 ± 0.3
5.0	16.2	3.2 ± 0.1

*Different concentrations of BHK-21 cells were incubated with a saturating amount of radiolabeled G protein at 25°C for 30 min. Radioactivity which remained associated with washed BHK-21 cells was evaluated and the number of G protein molecules bound per cell was calculated from the specific activity of the G protein.

Table 3. COMPETITIVE INHIBITION OF BINDING OF RADIOLABELED G PROTEIN BY UNLABELED G PROTEIN

Amount of G protein added (μg)	Number of labeled G protein molecules bound per cell*	Calculated binding† (% of control)	Observed binding‡ (% of control)
0 (control)	1.25×10^5	100	100
0.8	7.70×10^4	42	61
1.4	3.10×10^4	30	25
2.8	1.65×10^4	17	13

* Radiolabeled G protein (0.6 μg) was added to various concentrations of unlabeled G protein to 1×10^7 BHK-21 cells for 30 min at 25°C. The number of radiolabeled G protein molecules bound was determined from duplicate samples as described in the Methods.

† The calculated or expected binding was determined from the amount of G protein which should be bound to BHK-21 cells if the binding was saturable and if unlabeled G protein could competitively inhibit labeled G protein binding.

‡ The observed percentages of G protein binding were calculated from the amount bound compared to that bound in the controls with no unlabeled G protein added.

Table 4. EFFECTS OF ENZYME TREATMENT OF HOST CELLS ON ATTACHMENT OF G PROTEIN AND VSV*

enzymatic treatment of BHK-21 cells	binding component	$\frac{\text{treated}}{\text{untreated}} \times 100$ (%)
trypsin	G protein	107
	VSV	92
phospholipase A	G protein	136
	VSV	80
phospholipase C	G protein	81
	VSV	106

* Approximately 7.5 X 10^6 BHK-21 cells were treated with the various enzyme treatments as described in the Methods. Saturating amounts of G protein (8 μg) or 4,000 CPM of radiolabeled VSV were added to the treated cells and binding was determined relative to binding to untreated cells. The values presented are the average of duplicate measurements.

Table 5. EFFECTS ON THE BINDING OF G PROTEIN AND THE WHOLE VIRION WITH THE ADDITION OF UNLABELED VIRIONS OR G PROTEIN, RESPECTIVELY

Experiment 1. Competitive inhibition of viral attachment with G protein *

G protein added (μg)	Bound virions (CPM)	Percentage bound (%)
0.0 (control)	1086	100
0.5	1071	99
2.0	1240	114
10.0	1424	131
15.0	1192	110

Experiment 2. Competitive inhibition of G protein attachment with VSV†

Virus added (MOI)	G protein bound per cell	percentage bound (%)
0 (control)	3.3×10^5	100
10	3.1×10^5	94
100	3.7×10^5	112

* 3,000 CPM of whole VSV virions, mixed with various amounts of unlabeled G protein, were added to 7.5×10^6 BHK-21 cells and incubated at 25°C for 30 min. The radioactivity which remained with the washed cells was determined and compared to the amount of VSV which bound in the absence of G protein. Values represent the average of four measurements.

† Saturating amounts of radiolabeled G protein (9μg) were mixed with various amounts of unlabeled VSV prior to the addition to 7.5×10^6 BHK-21 cells. The number of G protein molecules bound was evaluated after 30 min incubation at 25°C. Values represent the average of four measurements.

ISOLATION OF THE GLYCOPROTEIN OF VESICULAR STOMATITIS VIRUS AND ITS BINDING TO CELL SURFACES

by

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B.S., Colorado State University, 1977

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ABSTRACT

The glycoprotein (G) of vesicular stomatitis virus was radiolabeled, extracted and purified to allow the study of its potential interaction with host cell surfaces. Incubation of BHK-21 cells with the radiolabeled viral glycoprotein resulted in a rapid attachment of the viral component to the cell surface. The attachment was shown to be temperature dependent and saturable in that approximately 3×10^5 molecules bound per cell. The deletion of Mg^{++} or Ca^{++} from the incubation medium had little affect on the glycoprotein binding. Treatment of the isolated G protein and intact virions with neuraminidase did not significantly decrease their binding to BHK-21 cells. Preincubation of cells with trypsin did not decrease the attachment of VSV virions nor the binding of purified G protein. Treatment of cells with phospholipase A or phospholipase C suggested that the binding of the glycoprotein and the intact virion may have been dissimilar. Unlabeled glycoprotein was shown to competitively inhibit binding of the labeled molecules although the presence of intact virions did not inhibit attachment of the G protein. Likewise, saturating amounts of the glycoprotein did not decrease binding of vesicular stomatitis virus to BHK-21 cells. These observations suggested that the isolated glycoprotein either bound to cell surface components that were distinct from the virion receptor or that the manner of the purified glycoprotein attachment was different than the G protein still associated with the intact virion. Chemical cross-linking and diagonal two-dimensional gel electrophoresis were used to identify and to compare the cell surface components responsible for glycoprotein and virion attachment.